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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6:
C12N 15/82, 5/10, A01H 5/00
A1
(11) International Publication Number: WO 97/06269
(43) International Publication Date: 20 February 1997 (20.02.97)

GB

(21) International Application Number: PCT/GB96/01883

(22) International Filing Date: 2 August 1996 (02.08.96)

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3 August 1995 (03.08.95)

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(30) Priority Data:

9515941.4

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Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: INDUCIBLE HERBICIDE RESISTANCE

(57) Abstract

The invention relates to DNA constructs which are capable of conferring on a plant inducible resistance to a herbicide. The inducible effect may be achieved by using a gene switch such as the *alcA/alcR* switch derived from *A. nidulans*. The invention relates in particular to inducible resistance to the herbicide N-phosphonomethyl glycine (glyphosate) and its salts.

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INDUCIBLE HERBICIDE RESISTANCE

The present invention relates to DNA constructs and plants incorporating them. In particular it relates to promoter sequences for the expression of genes which confer herbicide resistance on plants.

Recent advances in plant biotechnology have resulted in the generation of transgenic plants resistant to herbicide application. Herbicide tolerance has been achieved using a range of different transgenic strategies. One well documented example is the use the bacterial xenobiotic detoxifying gene phosphinothricin acetyl transferase (PAT) from Streptomyces hydroscopicus. Mutated genes of plant origin, for example the altered target site gene encoding acetolactate synthase (ALS) from Arabidopsis, have been successfully utilised to generate transgenic plants resistant to herbicide application. The PAT and ALS genes have been expressed under the control of strong constitutive promoter.

We propose a system where genes conferring herbicide tolerance would be expressed in an inducible manner dependent upon application of a specific activating chemical. This approach has a number of benefits for the farmer, including the following:

- Inducible control of herbicide tolerance would alleviate any risk of yield penalties
 associated with high levels of constitutive expression of herbicide resistance genes.
 This may be a particular problem as early stages of growth where high levels of
 transgene product may directly interfere with normal development. Alternatively high
 levels of expression of herbicide resistance genes may cause a metabolic drain for plant
 resources.
- The expression of herbicide resistance genes in an inducible manner allows the herbicide in question to be used to control volunteers if the activating chemical is omitted during treatment.
- The use of an inducible promoter to drive herbicide resistance genes will reduce the risk of resistant weed species becoming a major problem. If resistance genes were passed onto weed species from related crops, control could still be achieved with the herbicide in the absence of inducing chemical. This would particularly be relevant if the tolerance gene confirmed resistance to a total vegetative control herbicide which would be used (with no inducing chemical) prior to sowing the crop and potentially after the crop has been harvested. For example, it can be envisaged that herbicide

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resistance in cereals, such as wheat, might outcross into the weed wild oats or that herbicide resistance in oil seed rape or canola could be transferred to wild brassicas thus conferring herbicide resistance to these already troublesome weeds. A further example is that the inducible expression of herbicide resistance in sugar beet will reduce the risk of wild sugar beet becoming a problem.

Several gene regulation systems (gene switches) are known and may be used for conferring inducible herbicide resistance on plants. Many such gene switches are described in the review by Gatz (Current Opinion in Biotechnology (1996) 7, 168-172) and include systems such as the tetracycline repressor gene switch, the Lac repressor system, copper inducible systems such as that based on ACE 1, salicylic acid inducible promoters including the PR-1a system and systems based on sterioid hormones such as the glucocorticoid, progesterone and oestrogen receptor systems. Modifications of the glucocorticoid receptor systems which include the GAL 4 binding domain from yeast and the VP16 activator are described by Aoyama et al, The Plant Cell, (1995) 7, 1773-1785 and it is envisaged that similar systems may based on, for example insect steroid hormones rather than on mammalian steriod hormones. Indeed, a system based on the ecdysone receptor of Heliothis virescens has recently been described. Benzene sulphonamide gene switching systems are also known (Hershey et al, Plant Mol. Biol., 17, 679-690 (1991) as are systems based on the alcR protein from Aspergillus nidulans and glutathione S-transferase promoters.

Several genes which confer herbicide resistance are also known, for example, one herbicide to which resistance genes have been described and which is extremely widely used is N-phosphonomethyl-glycine (glyphosate) and its agriculturally acceptable salts including the isopropylamine, trimethylsulphonium, sodium, potassium and ammonium salts.

In a first aspect of the present invention there is provided a chemically inducible plant gene expression cassette comprising an inducible promoter operatively linked to a target gene which confers resistance to a herbicide.

Any herbicide resistance gene may be used but genes which confer resistance to N-phosphonomethyl-glycine or salts or derivatives thereof are especially preferred.

Several inducible promoters may be used to confer the inducible resistance and these include any of those listed above.

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However, a particularly useful gene switch for use in this area is based on the *alc* R regulatory protein from *Aspergillus nidulans* which activates genes expression from the *alc* A promoter in the presence of certain alcohols and ketones. This system is described in our International Patent Publication No. WO93/21334 which is incorporated herein by reference.

The alcA/alcR gene activation system from the fungus Aspergillus nidulans is also well characterised. The ethanol utilisation pathway in A. nidulans is responsible for the degradation of alcohols and aldehydes. Three genes have been shown to be involved in the ethanol utilisation pathway. Genes alcA and alcR have been shown to lie close together on linkage group VII and aldA maps to linkage group VIII (Pateman JH et al, 1984, Proc. Soc. Lond, B217:243-264; Sealy-Lewis HM and Lockington RA, 1984, Curr. Genet, 8:253-259). Gene alcA encodes ADHI in A. nidulans and aldA encodes AldDH, the second enzyme responsible for ethanol utilisation. The expression of both alcA and aldA are induced by ethanol and a number of other inducers (Creaser EH et al, 1984, Biochemical J, 255:449-454) via the transcription activator alcR. The alcR gene and a co-inducer are responsible for the expression of alcA and aldA since a number of mutations and deletions in alcR result in the pleiotropic loss of ADHI and aldDH (Felenbok B et al, 1988, Gene, 73:385-396; Pateman et al, 1984; Sealy-Lewis & Lockington, 1984). The ALCR protein activates expression from alcA by binding to three specific sites in the alcA promoter (Kulmberg P et al, 1992, J. Biol. Chem, 267:21146-21153).

The alcR gene was cloned (Lockington RA et al, 1985, Gene, 33:137-149) and sequenced (Felenbok et al, 1988). The expression of the alcR gene is inducible, autoregulated and subject to glucose repression mediated by the CREA repressor (Bailey C and Arst HN, 1975, Eur. J. Biochem, 51:573-577; Lockington RA et al, 1987, Mol. Microbiology, 1:275-281; Dowzer CEA and Kelly JM, 1989, Curr. Genet, 15:457-459; Dowzer CEA and Kelly JM, 1991, Mol. Cell. Biol, 11:5701-5709). The ALCR regulatory protein contains 6 cysteines near its N terminus co-ordinated in a zinc binuclear cluster (Kulmberg P et al, 1991, FEBS Letts, 280:11-16). This cluster is related to highly conserved DNA binding domains found in transcription factors of other ascomycetes. Transcription factors GAL4 and LAC9 have been shown to have binuclear complexes which have a cloverleaf type structure containing two Zn(II) atoms (Pan T and Coleman JE, 1990, Biochemistry, 29:3023-3029; Halvorsen YDC et al, 1990, J. Biol. Chem, 265:13283-13289). The structure of ALCR is

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similar to this type except for the presence of an asymmetrical loop of 16 residues between Cys-3 and Cys-4. ALCR positively activates expression of itself by binding to two specific sites in its promoter region (Kulmberg P et al, 1992, Molec. Cell. Biol, 12:1932-1939).

The regulation of the three genes, alcR, alcA and aldA, involved in the ethanol utilisation pathway is at the level of transcription (Lockington et al, 1987; Gwynne D et al, 1987, Gene, 51:205-216; Pickett et al, 1987, Gene, 51:217-226).

There are two other alcohol dehydrogenases present in A. nidulans. ADHII is present in mycelia grown in non-induced media and is repressible by the presence of ethanol. ADHII is encoded by alcB and is also under the control of alcR (Sealy-Lewis & Lockington, 1984). A third alcohol dehydrogenase has also been cloned by complementation with a adh-strain of S. cerevisiae. This gene alcC, maps to linkage group VII but is unlinked to alcA and alcR. The gene, alcC, encodes ADHIII and utilises ethanol extremely weakly (McKnight GL et al, 1985, EMBO J, 4:2094-2099). ADHIII has been shown to be involved in the survival of A. nidulans during periods of anaerobic stress. The expression of alcC is not repressed by the presence of glucose, suggesting that it may not be under the control of alcR (Roland LJ and Stromer JN, 1986, Mol. Cell. Biol, 6:3368-3372).

In summary, A. nidulans expresses the enzyme alcohol dehydrogenase I (ADH1) encoded by the gene alcA only when it is grown in the presence of various alcohols and ketones. The induction is relayed through a regulator protein encoded by the alcR gene and constitutively expressed. In the presence of inducer (alcohol or ketone), the regulator protein activates the expression of the alcA gene. The regulator protein also stimulates expression of itself in the presence of inducer. This means that high levels of the ADH1 enzyme are produced under inducing conditions (i.e. when alcohol or ketone are present). Conversely, the alcA gene and its product, ADH1, are not expressed in the absence of inducer. Expression of alcA and production of the enzyme is also repressed in the presence of glucose.

Thus the alcA gene promoter is an inducible promoter, activated by the alcR regulator protein in the presence of inducer (i.e. by the protein/alcohol or protein/ketone combination). The alcR and alcA genes (including the respective promoters) have been cloned and sequenced (Lockington RA et al, 1985, Gene, 33:137-149; Felenbok B et al, 1988, Gene, 73:385-396; Gwynne et al, 1987, Gene, 51:205-216).

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Alcohol dehydrogenase (adh) genes have been investigated in certain plant species. In maize and other cereals they are switched on by anaerobic conditions. The promoter region of adh genes from maize contains a 300 bp regulatory element necessary for expression under anaerobic conditions. However, no equivalent to the alcR regulator protein has been found in any plant. Hence the alcR/alcA type of gene regulator system is not known in plants.

Constitutive expression of alcR in plant cells does not result in the activation of endogenous adh activity.

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According to a second aspect of the invention, there is provided a chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to an alcR regulator sequence which encodes an alcR regulator protein, and an inducible promoter operatively linked to a target gene which confers herbicide resistance, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.

The inducible promoter is preferably derived from the alcA gene promoter but may, alternatively be derived from alcR, aldA or other alcR-induced genes.

We have found that the alcA |alcR switch is particularly suited to drive herbicide tolerance genes for at least the following reasons.

- 1. The alcA/alcR switch has been developed to drive high levels of gene expression. In addition, the regulatory protein alcR is preferably driven from a strong constitutive promoter such as polyubiquitin. High levels of induced transgene expression, comparable to that from a strong constitutive promoter, such as 35 CaMV, can be achieved.
- 2. If a gene switch is to be used in a situation where the activating chemical is applied simultaneously with the herbicide, a rapid elevation in the levels of herbicide resistance gene is required. Figure 1 reveals a time course of marker gene expression (CAT) following application of inducing chemical. This study shows a rapid increase (2 hours) of CAT expression following foliar application of inducing chemical. The immediate early kinetics of induction are brought about be expressing the regulatory protein in constitutive manner, therefore no time lag is encountered while synthesis of transcription factors takes place. In addition we have chosen a simple two component system which does not rely on a complex signal transduction system.

3. We have tested the specificity of alcA/alcR system with a range of solvents used in agronomic practice. A hydroponic seedling system revealed that ethanol, butan-2-ol and cyclohexanone all gave high levels of induced reporter gene expression (Figure 2). In contrast when the alcohols and ketones listed in Table 1 in which are used in agronomic practice were applied as a foliar spray only ethanol gave high levels of induced reporter gene activity (Figure 3).

Table 1

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1.	Isobutyl methyl ketone	13.	acetonyl acetone
2.	Fenchone	14.	JF5969 (cyclohexanone)
3.	2-heptanone	15.	N-methyl pyrrolidone
4.	Di-isobutyl ketone	16.	polyethylene glycol
5.	5-methyl-2-hexanone	17.	propylene glycol
6.	5-methylpentan-2,4-diol	18.	acetophenone
7.	ethyl methyl ketone	19.	JF4400 (methylcyclohexanone)
8.	2-pentanone	20.	propan-2-ol
9.	glycerol	21.	butan-2-ol
10.	γ-butyrolactone	22.	acetone
11.	diacetone alcohol	23.	ethanol
12.	tetrahydrofurfuryl alcohol	24.	dH₂O

This is of significance since illegitimate induction of transgenes will not be encountered by chance exposure to formulation solvents. Ethanol is not a common component of agrochemical formulations and therefore with appropriate spray management can be considered as a specific inducer of the *alc* A / R gene switch in a field situation.

4. A range of biotic and abiotic stresses for example pathogen infection, heat, cold, drought, wounding, flooding have all failed to induce the alcA /alcR switch. In addition a range of non-solvent chemical treatments for example salicylic acid, ethylene, absisic acid, auxin, gibberelic acid, various agrochemicals, all failed to induce the alcA / alcR system.

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The first promoter may be constitutive or tissue-specific, developmentally-programmed or even inducible. The regulator sequence, the *alcR* gene, is obtainable from *Aspergillus nidulans*, and encodes the *alcR* regulator protein.

The inducible promoter is preferably the alcA gene promoter obtainable from Aspergillus nidulans or a "chimeric" promoter derived from the regulatory sequences of the alcA promoter and the core promoter region from a gene promoter which operates in plant cells (including any plant gene promoter). The alcA promoter or a related "chimeric" promoter is activated by the alcR regulator protein when an alcohol or ketone inducer is applied.

The inducible promoter may also be derived from the aldA gene promoter, the alcB gene promoter or the alcC gene promoter obtainable from Aspergillus nidulans.

The inducer may be any effective chemical (such as an alcohol or ketone). Suitable chemicals for use with an *alcA/alcR*-derived cassette include those listed by Creaser *et al* (1984, Biochem J, 225, 449-454) such as butan-2-one (ethyl methyl ketone), cylcohexanone, acetone, butan-2-ol, 3-oxobutyric acid, propan-2-ol, ethanol.

The gene expression cassette is responsive to an applied exogenous chemical inducer enabling external activation of expression of the target gene regulated by the cassette. The expression cassette is highly regulated and suitable for general use in plants.

The two parts of the expression cassette may be on the same construct or on separate constructs. The first part comprises the regulator cDNA or gene sequence subcloned into an expression vector with a plant-operative promoter driving its expression. The second part comprises at least part of an inducible promoter which controls expression of a downstream target gene. In the presence of a suitable inducer, the regulator protein produced by the first part of the cassette will activate the expression of the target gene by stimulating the inducible promoter in the second part of the cassette.

In practice the construct or constructs comprising the expression cassette of the invention will be inserted into a plant by transformation. Expression of target genes in the construct, being under control of the chemically switchable promoter of the invention, may then be activated by the application of a chemical inducer to the plant.

Any transformation method suitable for the target plant or plant cells may be employed, including infection by Agrobacterium tumefaciens containing recombinant Ti

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plasmids, electroporation, microinjection of cells and protoplasts, microprojectile transformation and pollen tube transformation. The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both transformed monocot and dicot plants may be obtained in this way.

Examples of genetically modified plants which may be produced include field crops, cereals, fruit and vegetables such as: canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion.

The invention further provides a plant cell containing a gene expression cassette according to the invention. The gene expression cassette may be stably incorporated in the plant's genome by transformation. The invention also provides a plant tissue or a plant comprising such cells, and plants or seeds derived therefrom.

The invention further provides a method for controlling plant gene expression comprising transforming a plant cell with a chemically-inducible plant gene expression cassette which has a first promoter operatively linked to an alcR regulator sequence which encodes an alcA regulator protein, and an inducible promoter operatively linked to a target gene which confers herbicide resistance, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.

This strategy of inducible expression of herbicide resistance can be achieved with a pre-spray of chemical activator or in the case of slow acting herbicides, for example N-phosphonomethyl-glycine (commonly known as glyphosate), the chemical inducer can be added as a tank mix simultaneously with the herbicide.

This strategy can be adopted for any resistance conferring gene/corresponding herbicide combination. For example, the *alcA/alcR* gene switch can be used with:

- Maize glutathione S-transferase (GST-27) gene (see our International Patent Publication No WO90/08826), which confers resistance to chloroacetanilide herbicides such as acetochlor, metolachlor and alachlor.
- Phosphinotricin acetyl transferase (PAT), which confers resistance to the herbicide commonly known as glufosinate.

- Acetolactate synthase gene mutants from maize (see our International Patent
 Publication No WO90/14000) and other genes, which confer resistance to sulphonyl
 urea and imadazlonones.
- Genes which confer resistance to glyphosate. Such genes include the glyphosate oxidoreductase gene (GOX) (see International Patent Publication No. WO92/00377 in the name of Monsanto Company); genes which encode for 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS), including Class I and Class II EPSPS, genes which encode for mutant EPSPS, and genes which encode for EPSPS fusion peptides such as that comprised of a chloroplast transit peptide and EPSPS (see for example EP 218 571, EP 293 358, WO91/04323, WO92/04449 and WO92/06201 in the name of Monsanto Company); and genes which are involved in the expression of CPLyase, Various further preferred features and embodiments of the present invention will now be described in the non-limiting examples set out below and with reference to the drawings in

Figure 1 illustrates the time course of marker gene expression (CAT) following application of inducing chemical;

Figure 2 illustrates the levels of induced reporter gene expression on root drenching with a range of solvents;

Figure 3 illustrates the levels of induced reporter gene activity when the chemicals listed in Table 1 were applied as a foliar spray;

Figure 4 illustrates the production of the 35S regulator construct by ligation of *alc*R cDNA into pJR1.

Figure 5 illustrates the production of the reporter construct;

Figure 6 is a summary of the cassettes and specific plant transformation constructs;

Figure 7 illustrates the chloroplast transit sequence 1 from Arabidopsis RUBISCO (CPT 1);

Figure 8 shows the sequence of plasmid pMJB1;

Figure 9 is a map of plasmid pJRIi;

which:

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Figure 10 illustrates the chloroplast transit sequence CTP2 from EPSPS class I gene from Petunia hybrida;

Figure 11 is a map of plasmid pUB-1;

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Figure 12 is a map of plasmid pMF6;

Figure 13 is a map of plasmid pIE109 in which the numbers are in base pairs (not to scale) and the following abbreviations are used:

ADH_i Alcohol dehydrogenase from maize;

PAT Phosphinothricin acetyl transferase (Basta resistance gene);

AMP Ampicillin resistance gene;

CaMV 35S Cauliflower mosaic virus 35S promoter;

nos Poly A Nopaline synthase poly A region;

ori ColE1 origin of replication from pUC

Figure 14 is a map of plasmid pMV1 in which the numbers are in base pairs (not drawn to scale) and the abbreviations are as for Figure 13 with the following additional abbreviations:

UBQ_p Maize ubiquitin promoter;

UBQ_i Maize ubiquitin intron;

nos Nopaline synthase 3' terminator;

CZP1 GOX Chloroplast transit peptide - glyphosate oxidase sequence;

CZP2 GPSPS Chloroplast transit peptide - EPSP synthetase sequence;

Figure 15 shows the preparation of plasmid pUC4 by ligation of pAr3 and pBSSK; Figure 16 is a map of plasmid pMV2 in which the numbers are in base pairs (not drawn to scale) and the abbreviations are as for Figure 14 with the following

additional abbreviations:

AlcA Aspergillus nidulans alcA promoter;

AlcR Aspergillus nidulans alcR promoter;

Figure 17 is a map of plasmid pDV1-pUC;

Figure 18 is a map of plasmid pDV2-pUC;

Figure 19 is a map of plasmid pDV3-Bin;

Figure 20 is a map of plasmid pDV4-Bin; and

Figure 21 is a western Blot showing the expression of EPSPS and GOX in transformants.

EXAMPLES

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We have chosen to exemplify the *alcA/alcR* gene switch with genes conferring resistance to glyphosate. The switch will be used to drive inducible expression of glyphosate oxidase (GOX) in plants. Switchable GOX has been expressed alone or in conjunction with constitutive expression of 5-enol-pyruvylshikimate 3-phosphate (EPSPS) CP4. Constructs have been optimised for expression in monocotyledonous and dicotyledonous crop species. EXAMPLE 1

Production Of The alcR Regulator Construct.

The alcR genomic DNA sequence has been published, enabling isolation of a sample of alcR cDNA.

The alcR cDNA was cloned into the expression vectors pJR1(pUC). pJR1 contains the Cauliflower Mosaic Virus 35S promoter. This promoter is a constitutive plant promoter and will continually express the regulator protein. The nos polyadenylation signal is in the expression vector.

Figure 4 illustrates the production of the 35S regulator construct by ligation of alcR cDNA into pJR1. Partial restriction of the alcR cDNA clone with BamHI was followed by electrophoresis in an agarose gel and the excision and purification of a 2.6 Kb fragment. The fragment was then ligated into the pJR1 vector which had been restricted with BamHI and phosphatased to prevent recircularisation. The alcR gene was thus placed under control of the CaMV 35S promoter and the nos 3' polyadenylation signal in this "35S-alcR" construct. EXAMPLE 2

Production Of The alcA-CAT Reporter Construct Containing The Chimeric Promoter.

The plasmid pCaMVCN contains the bacterial chloramphenicol transferase (CAT) reporter gene between the 35S promoter and the *nos* transcription terminator (the "35S-CAT" construct).

The alcA promoter was subcloned into the vector pCaMVCN to produce an "alcA-CAT" construct. Fusion of part of the alcA promoter and part of the 35S promoter created a chimeric promoter which allows expression of genes under its control.

Figure 5 illustrates the production of the reporter construct. The alcA promoter and the 35S promoter have identical TATA boxes which were used to link the two promoters together using a recombinant PCR technique: a 246 bp region from the alcA promoter and

the 5' end of the CAT gene from pCaMVCN (containing part of the -70 core region of the 35S promoter) were separately amplified and then spliced together using PCR. The recombinant fragment was then restriction digested with BamHI and HindIII. The pCaMVCN vector was partially digested with BamHI and HindIII, then electrophoresed so that the correct fragment could be isolated and ligated to the recombinant fragment.

The ligation mixtures were transformed into *E coli* and plated onto rich agar media. Plasmid DNA was isolated by miniprep from the resultant colonies and recombinant clones were recovered by size electrophoresis and restriction mapping. The ligation junctions were sequenced to check that the correct recombinants had been recovered.

EXAMPLE 3

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Glyphosate Resistance Constructs

A summary of the cassettes and specific plant transformation constructs is shown in Figure 6.

Dicot Vector 1

Vector 1 is a constitutive control plasmid containing the glyphosate oxidase gene (GOX) fused to the chloroplast transit sequence 1 from Arabidopsis RUBISCO (CPT 1) (Figure 7) driven by the enhanced 35S CaMV promoter (ES) and the TMV omega translational enhancer sequence (TMV). Vector 1 utilizes the nopoline synthase terminator (nos). The synthetic GOX gene with the addition of CTP 1 was synthesised with information from patent publication WO92/00377 with addition of NcoI site at the translation start ATG, and a Kpn I at the 5' end. Internal Sph I sites and NcoI site were deleted during synthesis with no change in amino acid usage. The CTP 1 GOX synthesised sequence was isolated as a Nco I Kpn I fragment and ligated using standard molecular cloning techniques into NcoI KpnI cut pMJB1, a plasmid based on pIBT 211 containing the CaMV 35 promoter with duplicated enhancer linked to the tobacco mosaic virus translational enhancer sequence replacing the tobacco etch virus 5' non-translated leader, and terminated with the nopaline synthase poly (A) signal (nos) (Figure 8).

A cassette containing enhanced 35 CaMV TMV sequence CTP1 GOX and nos terminator (dicot vector 1 pUC Figure 17) was isolated as a *HindIII EcoRI* fragment and ligated into *Hind III EcoRI* cut pJRIi, a Bin 19 base plant transformation vector (Figure 9).

WO 97/06269

Dicot Vector 2

The synthetic EPSPS CP4 gene, fused to the chloroplast transit sequence CTP2 (Figure 10) from EPSPS class I gene from Petunia hybrida, was synthesised with data from patent WO 92/04449 with NcoI at the translation initiation ATG. A internal Sph I site was silenced in the EPSPS CP4 gene with no change of amino acid usage.

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A fragment containing the synthetic CTP 2 CP4 EPSPS was isolated as a Ncol Sac I fragment and ligated in to pMJBI. A fragment containing the CaMV 35 promoter with a duplicated enhancer, TMV omega sequence CTP 2 transit peptide, EPSPS and nos terminator was isolated as a EcoRI Hind III fragment (dicot vector 2 pUC Figure 18) and cloned into pJRIi to give dicot vector 2 pUC (Figure 18).

Upon sequencing the junctions of dicot vector 2, an additional sequence was identified inserted between the SacI site and the beginning of the nos terminator. This was as follows:

5' AGG CTG CTT GAT GAG CTC GGT ACC CGG GGA TCC ATG GAG CCG AAT 3'

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Dicot Vector 3

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A control vector with both EPSPS and GOX genes was constructed by cutting dicot vector 2 with EcoRI and inserting an ΔEcoRI Sph I ΔEcoRI linker. The sequence of the linker is shown below:

5' AAT TAG GGG CAT GCC CCT 3'

The resultant vector was cut with Sph I to liberate the cassette B which was cloned into an SphI site in dicot vector 1), 5' to the 35 CaMV promoter. Cassettes 1) and 2) were then excised as a HindIII and EcoRI fragment from dicot vector 3- pUC (Figure 19) and cloned in to pJRIi.

25 Dicot Vector 4

An inducible GOX vector was constructed by excising the CAT gene from "palcCAT" as PstI fragment. The vector band, containing the alcA promoter and nos terminator was gel purified and used in ligations with a PstI-XhoI-KpnI-PstI linker, the sequence of which is as follows:

5' GCC ACT CGA GCT AGG TAC CCT GCA 3'

The orientation of this was confirmed by sequence analysis. The TMV omega and CTPI GOX sequence from dicot vector 1) were isolated as a XhoI KpnI fragment and cloned into the alcA nos vector containing the XhoI-KpnI-PstI linker. The alcA TMV CTP1 GOX

PCT/GB96/01883

nos cassette was excised as a *Hin*dIII fragment and cloned into the plant transformation vector "p35S-alc R", containing the alcR cDNA nos terminator under the control of the 35 CaMV promoter to form dicot vector 4 (Figure 20).

Dicot Vector 5

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Dicot vector 5 (Figure 22) containing inducible GOX and constitutive EPSPS genes was prepared using the following cloning strategy. Dicot vector 2 (pDV2 -pUC) was modified by cloning in a Δ*Eco*RI-*Hin*dIII-Δ*Eco*RI linker into the *Eco*RI site to allow excision of the CaMV en-CTP2-EPSPS -nos cassette as a *Hin*dIII fragment. This fragment was then ligated into *Hin*dIII cut pDV4-Bin. Recombinants containing all three cassettes ie 35S-*Alc*R, CaMVen-CTP2-EPSPS-nos and AlcA-CTP1-GOX-nos were selected by hybridization with radiolabelled oligonucleotides. Confirmation of orientation was done by sequencing across all borders.

Monocot Vectors

Vector 1: Cassette D

15 An EcoRI-NotI-EcoRI linker (5'AATTCATTTGCGGCCGCAAATG3') was inserted into dicot vector pDV1. The plasmid was cut with NcoI and the 5' overhang filled-in with DNA Polymerase I Klenow fragment. The linear vector was then cut with NotI and the resulting blunt/NotI fragment containing the CTP1 GOX and nos terminator was ligated into a SmaI/NotI digested pPUB1 vector (Figure 12) containing the polyubiquitin promoter, 20 polyubiquitin intron with а KpnI-NotI-KpnI linker (5'CATTTGCGGCCGC AAATGGTAC3') insertion. A HindIII-NotI-HindIII linker (5'AGCTTGCAGCGGC CGCTGCA3') was inserted into the resulting construct.

Vector 1: Cassette E

An EcoRI-NotI-EcoRI linker (5'AATTCATTTGCGGCCGCAAATG3') was inserted into dicot vector pDV2. The plasmid was cut with NcoI and the 5' overhang filled-in with DNA Polymerase I Klenow fragment. The linear vector was then cut with NotI and the resulting blunt/NotI fragment containing the CTP2 EPSPS and nos terminator was ligated into a SmaI/NotI digested pPUB1 vector containing the polyubiquitin promoter, polyubiquitin intron with a KpnI-NotI-KpnI linker (5'CATTTGCGGCCGCAAATGGT AC3') insertion to create plasmid 1. The PAT selectable marker cassette (35S CaMV promoter, AdhI intron,

phosphinothricin acetyl transferase gene (PAT), nos terminator) was excised from pIE108 (Figure 14) and cloned into the *HindIII* site on plasmid 1 to give mononcot cassette E. Diagnostic restriction digestion was used to confirm that the selectable marker cassette was inserted 5' to 3' in the same orientation as the CTP2 EPSPS cassette.

A fragment containing the polyubiquitin promoter, polyubiquitin intron, CTP1 GOX and nos terminator was excised from cassette D with *NotI* and ligated into *NotI* cassette E to form monocot vector 1 (Figure 14). Restriction digestion was used to confirm that the two cassettes were inserted in the same orientation.

The selectable marker cassette (35 CaMV promoter, AdhI intron, phosphinothricin acetyl transferase gene (PAT), nos) was excised from pIE108 and cloned into the Hind III site in 5) to give monocot cassette E.

Vector 1

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A fragment containing the polyubiquitin promoter, polyubiquitin intron GOX and nos was existed from cassette D with *NotI* and cloned into *NotI* cut casette E, to form monocot vector 1.

Vector 2 Cassette F

An EcoRI fragment from pUC4 (Figure 15) containing the alcR cDNA and nos terminator sequences was blunt end-filled with DNA Polymerase I Klenow fragment, ligated into pUB1 with the KpnI-NotI-KpnI linker insertion and orientated by restriction analysis. The PAT selectable marker cassette was inserted in the HindIII site after excision from pIE108 and orientated by restriction analysis to create vector 1. Plasmid 1 above containing the polyubiquitin promoter, polyubiquitin intron, CTP2 EPSPS and nos terminator was cut with HindIII and a ΔHindIII-NotI-HindIII linker:

5' AGCTCGCAGCGGCCGCTGCA3'

5'GCGTCGCCGGCGACGTTCGA3'

inserted and orientated by sequencing to create vector 2.

A ClaI-NcoI-ClaI linker (5'CGATGCAGCCATGGCTGCAT3') was inserted into pMF6 (Figure 13) to give vector 3. An NcoI/KpnI fragment containing CTP1 GOX was excised from pDV1 and inserted into NcoI/KpnI cut vector 3 to create vector 4. A SalI fragment containing the maize AdhI intron, CTP1 GOX was excised from vector 4 and ligated into SalI cut pUC2 containing the alcA promoter and nos terminator and orientated by sequencing to create vector 5. A HindIII fragment from vector 5 containing the alcA promoter, maize AdhI

intron, CTP1 GOX and nos terminator was ligated into HindIII cut vector 2 and orientated by restriction digestion. A NotI fragment from the resulting construct containing polyubiquitin promoter, polyubiquitin intron, CTP2 EPSPS, nos terminator, alcA promoter, maize AdhI intron, CTP1 GOX and nos terminator was ligated into NotI cut vector 1 and orientated by restriction analysis to create monocot vector 2 (Figure 16).

EXAMPLE 4

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Plant Transformation

Plasmids for dicot transformation were transferred to Agrobacterium tumefaciens LBA4404 using the freeze thaw method described by Holsters et al 1978.

Tobacco transformants were produced by the leaf disc method described by Bevan 1984. Shoots were regenerated on a medium containing 100 mg/l kanamycin. After rooting plants were transferred to the glasshouse and grown under 16h light/8h dark conditions.

Oilseed rape (Brassica napus cv westar) transformations were performed using the cotyledon petiole method described by Moloney et al 1989. Selection of transformed material was performed on kanamycin (15 mg/l). Rooted shoots were transferred directly to a soil based compost and grown to maturity under controlled glasshouse conditions (16h day 20°C day, 15°C night 60% RH).

Maize transformation was performed using the particle bombardment approach as described by Klein et al 1988. Selections were performed on 1 mg/l biolophos.

Sugar beet transformation was performed using the guard cell protoplast procedure see our International Patent Publication No. WO95/10178.

Results showing details of the transgenic plants obtained are shown in Tables 2 and 3 below.

Table 2 - Transformation Details For Tobacco

Vector	Species	Shoots removed	Rooted	
pDV1	Tobacco	150	57	
pDV2	Tobacco	150	60	
pDV3	Tobacco	270	77	
pDV4	Tobacco	350	135	
pDV5	Tobacco	150	75	

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Table 3 - Transformation Details in Oil Seed Rape

	Vector	Species	Shooting Calli	Rooted
	pDV1	OSR	14	shoots from 14
	pDV2	OSR	13	shoots from 13
5	pDV3	OSR	18	shoots from 18
	pDV4	OSR	20	shoots from 20
	pDV5	OSR	19	shoots from 18

EXAMPLE 5

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10 Transgenic Plant Analysis

Polymerase Chain Reaction (PCR)

Genomic DNA for PCR analysis of transgenic plants was prepared according to the method described by Edwards *et al* 1992. PCR was performed using conditions described by Jepson *et al*, Plant Molecular Biology Reporter, 9(2), 131-138 (1991). Primer sets were designed for each of the introduced cassettes.

The plants were analysed using the following oligonucleotide combinations:-

pDV1 TMV1 + GOX1, GOX3 + nos1

pDV2 TMV1 + EPSPS1, EPSPS3 + nos1

pDV3 EPSPS3 + GOX1

20 pDV4 35S + AlcR1, AlcA2 + GOX1

pDV5 35S + AlcR1, AlcA2 + GOX1, TMV1 + EPSPS1

Oligonucleotide sequences are given below:-

	TMV1	5′	CTCGAGTATTTTTACAACAATTACCAAC
25	GOX1	5 ′	AATCAAGGTAACCTTGAATCCA
	GOX3	5′	ACCACCAACGGTGTTCTTGCTGTTGA
	NOS1	5′	GCATTACATGTTAATTATTACATGCTT
	EPSPS1	5′	GTGATACGAGTTTCACCGCTAGCGAGAC
	EPSPS3	5′	TACCTTGCGTGGACCAAAGACTCC
30	35 <i>s</i>	5′	GTCAACATGGTGGAGCACG
	AlcR1	5′	GTGAGAGTTTATGACTGGAGGCGCATC
	AlcA2	5′	GTCCGCACGGAGAGCCACAAACGA

Selection on Glyphosate

Kill Curves for Tobacco var Samsun and Brassica napus var Westar on glyphosate

Both species were tested on a range of glyphosate concentrations by inserting, in the case of tobacco a 5-6mm stem segment carrying a leaf node and in the case of oil seed rape the growing tip plus two leaves into MS medium containing glyphosate at 0, 0.0055, 0.011, 0.0275, 0.055 and 0.01 mM glyphosate isopropylamine salt. The results were scored after two weeks growth as and are given in Table 4 below.

Table 4

10	Conc	Westar	Tobacco
	0 -	Good stem growth, 4-5 new leaves, roots up to 5cm	As OSR
	0.005	No stem growth, 1 new leaf, roots to 1cm	No growth in any
			organ
	0.011	No stem growth, no new leaves, roots~0.5cm	. 67
15	0.0275	No stem growth, no new leaves, roots~2mm	67
	0.055	No growth in any organ, ends of stem blackened	67
	0.01	As for 0.055mM	"

Selection for glyphosate tolerant transformants was performed on glyphosate concentrations of 0.01 and 0.05mM.

Constitutively tolerant plants

Following from the data obtained on wild type plants, pDV1,2 and 3 PCR +ve primary transformants were screened on MS medium containing glyphosate at the levels described above. For tobacco this was done by inserting three or four stem sections per transformant into the medium and using untransformed Samsun as control. Scoring was based on the behaviour of the majority. Plants showing tolerance at the higher concentration of herbicide were grown on to maturity in the glass house, for seed collection.

Segregation Test

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Seeds were sterilized in 10% bleach for 10 min. After several washes in sterile water 200 seeds were sown on 1/2 MS medium (2.3 g/l MS salt, 1.5% sucrose, 0.8% Bactoagar,

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pH 5.9) containing 100 mg/l kanamycin. Seeds were grown at 26°C with 16 hours/8 hours light/dark prior to scoring.

Western Analysis

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Antibody Generation

GOX and EPSPS protein were over expressed in E.Coli using a pET expression system. Following IPTG induction GOX and EPSPS were electro eluted from the shake flask grown cell paste and used to immunise rabbits (two animals per clone).

Preparation of Tissue Extracts for Immunoblotting

120 mg of leaf tissue plus 60 mg PVPP and 500 µl extraction buffer (50 mM Tris-HCl pH 8, 1 mM EDTA, 0.3 mM DTT) were ground with a blender for several minutes. After homogenisation the extract was centrifuged at 15,000 rpm for 15 min. The supernatant was stored at -80° C until required. Protein concentrations in the extract were measured according to Bradford.

SDS-PAGE and Immunoblotting

 $25~\mu g$ protein were separated by SDS-PAGE. The running buffer was 14.4 % (w/v) glycine, 1 % (w/v) SDS and 3 % (w/v) Tris Base. The samples were loaded according to Laemmli.

After SDS-PAGE proteins were electroblotted overnight with 40 mA onto nitrocellulose (HybondTM C, Amersham) using an electroblot unit from Biorad. The membrane was stained in 0.05 % CPTS dissolved in 12 mM HCl. Blots were rinsed in 12 mM HCl and destained for 5 - 10 min in 0.5 M NaHCO₃ followed by an intensive rinse with H₂O. Membranes were then blocked, immunodetected and washed according to the Amersham ECL kit. Indirect immunodetections were performed with a 1:10000 dilution of a rabbit anti-GOX or anti-EPSPS polyclonal as first antibody and with a 1:1000 dilution of an anti-rabbit second antibody, associated with horseradish peroxidase. An additional wash was carried out overnight to eliminate background. Detection was performed using the ECL kit from Amersham and the results are shown in Figure 21 in which Lane (1) is the control and the remaining lanes are transformants. The western analysis demonstrates that some transformants are capable of expressing GOX and EPSPS.

Constitutively tolerant plants

Cell extracts were prepared from each glyphosate tolerant plant and the amount of
expressed protein estimated by western analysis using antibody appropriate to the

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transformant. Plants expressing very high levels of GOX or EPSPS were tested on higher levels of glyphosate to relate level of expression to herbicide tolerance.

Inducibly tolerant plants

To demonstrate inducible tolerance to glyphosate PCR positive primary transformants from the transformations with pDV4 and 5 were transferred directly to the glass house. After two weeks the plants were induced by an ethanol root drench (5% solution) and left for 24 hours prior to western analysis performed to assess level of expression of GOX after induction. After a period of time to allow the plants to return to the uninduced state, the western analysis was repeated to allow selection of inducibly tolerant plants. Plants which showed the highest levels of GOX expression following ethanol treatment were taken forward to time course analysis. GOX levels were assessed at 6, 12, 18, 24, 36, 48 hours following ethanol treatment, by western analysis.

High expressing GOX plants for both pDV4 and pDV5 were used in glass house trials to demonstrate inducible glyphosate tolerance. Plants were induced using a range of ethanol concentrations (1-15%) by root drench application to pot grown plants. Following GOX induction plants were sprayed with glyphosate. Wild type controls and uninduced plants were also treated with herbicide.

Northern Analysis

Primary transformants containing dicots vector 2)., 3)., and 5). were analysed by northern blot analysis - using a CTP2 EPSPS probe as a *NcoI Sac* I fragment. Primary transformants containing the dicot vectors 1). 3). were analysed by northern blotting using a CTPI GOX probe as a *NcoI KpnI* fragment. Similarly, transgenic corn lines containing monocot vectors 1). and 2). were analysed using a CTP2 EPSPS probe.

Transformants containing dicot vector 5). or monocot vector 2). were treated with a foliar application of 5% ethanol to induce GOX levels. RNA was isolated 24 hours after treatment and subjected to northern analysis with a CTPI GOX probe.

Primary transformants which were PCR positive for the appropriate cassettes and showed GOX or EPSPS transcript levels were taken for further analysis.

Glyphosate Oxidoreductase Assay

Assays for glyphosate oxidoreductase were carried out as described by Kishore and Barry (WO 92/00377). These entailed measuring glyphosate - dependent uptake of oxygen

using an oxygen electrode, detection of glyoxylate formation by reaction with 2, 4 - dinitrophenylhydrazine and determination of the hydrozone using HPLC or, preferably, using [3-14C] - glyphosate as the substrate and detecting the formation of radioactive aminomethyl phosphonic acid via HPLC on an anion exchange column.

5 EPSPS Assay

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Assays for 5-enol-pyruvylshikimate-3-phosphate (EPSP) synthase activity in plant extracts were carried out (1) by following the disappearance of the phosphoenol pyruvate substrate (as described by Rubin, J.L., Gaines, C.G and Jensen, R.A., in Plant Physiol (1984 75, 839-845) or (2) by conducting the assay in the reverse direction and coupling to pyruvate kinase and lactate dehydrogenase (as described by Mousdale D.M. and Coggins J.R. in Planta (1984) 160, 78-83) or (3) by using 14(-labelled phosphoenol pyruvate as substrate and detecting the formation of radioactive EPSP by HPLC on an anion exchange column and detecting using a flow-through radioactivity detector as described by Della-Cioppa et al in Proc. Nat. Acad. Sci. (USA) (1986), 83, 6873-6877. The latter assay was used to confirm that the EPSP synthase activity was, as expected, relatively resistant to inhibition by glyphosate.

CLAIMS

- A chemically inducible plant gene expression cassette comprising an inducible promoter operatively linked to a target gene which confers resistance to a herbicide.
- A chemically inducible plant gene expression cassette as claimed in claim 1, wherein the herbicide is N-phosphonomethyl-glycine or a salt or derivative thereof.
- 3. A chemically inducible plant gene expression cassette as claimed in claim 1 or claim 2, wherein the inducible promoter is the tetracycline repressor gene switch, the Lac repressor system, a copper inducible systems such as that based on ACE 1, a salicylic acid inducible promoters, for example the PR-1a system, a system based on a sterioid hormone such as the glucocorticoid, progesterone and oestrogen receptor systems or a modification of one of these such as a glucocorticoid receptor system which includes the GAL 4 binding domain from yeast and the VP16 activator, an insect steroid hormones systems such as that based on the ecdysone receptor of Heliothis virescens, a benzene sulphonamide gene switching system, a gene switching based on the alcR protein from Aspergillus nidulans or a glutathione S-transferase promoter.
- 4. A chemically inducible plant gene expression cassette comprising a first promoter operatively linked to an alcR regulator sequence which encodes an alcR regulator protein, and an inducible promoter operatively linked to a target gene which confers herbicide resistance, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.
 - 5. A plant gene expression cassette according to claim 4, wherein the inducible promoter is derived from the alcA, alcR, aldA or other alcR-induced gene promoter.
- A plant gene expression cassette according to either of claims 4 and 5, wherein the inducible promoter is a chimeric promoter.

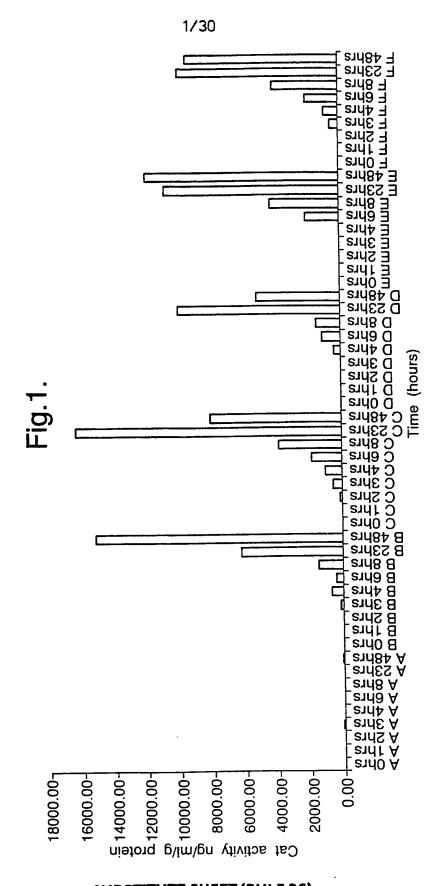
7. A plant gene expression cassette according to any preceding claim, wherein the target gene confers resistance to the herbicide N-phosphonomethyl-glycine or a salt or derivative thereof.

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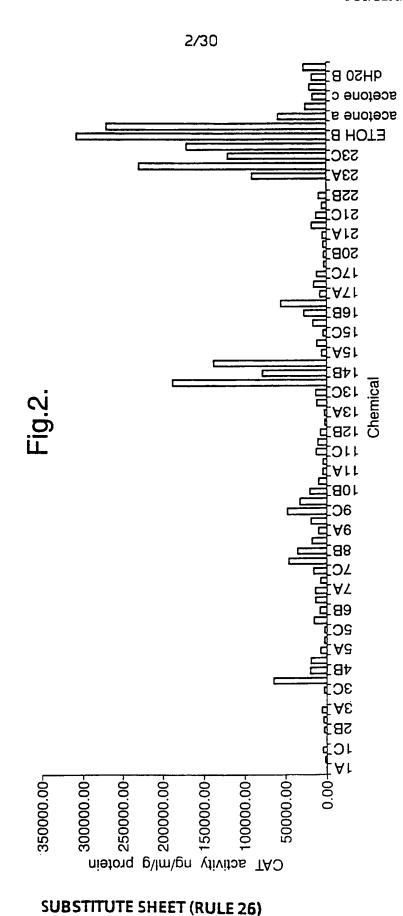
- 8. A plant cell containing a plant gene expression cassette according to any preceding claim.
- 9. A plant cell according to claim 8, wherein the plant gene expression cassette is stably incorporated in the plant's genome.
 - 10. A plant tissue comprising a plant cell according to either of claims 8 and 9.
 - 11. A plant comprising a plant cell according to either of claims 8 and 9.

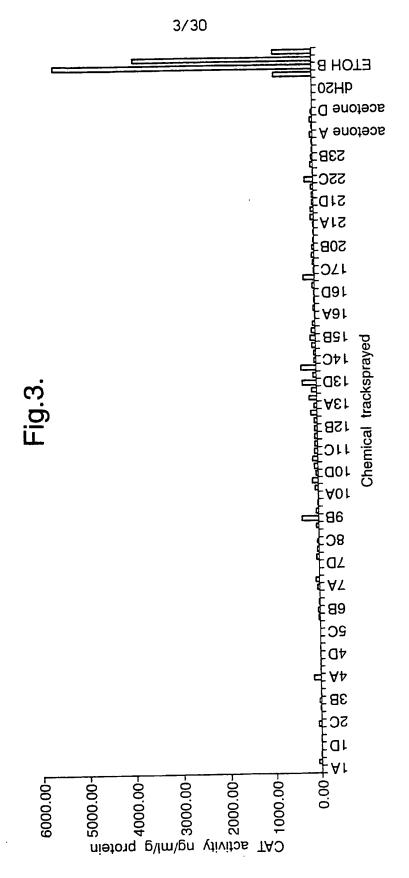
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- 12. A plant derived from a plant according to claim 11.
- 13. A seed derived from a plant according to either of claims 11 and 12.
- 20 14. A method of controlling herbicide resistance comprising transforming a plant cell with the plant gene expression cassette of any one of claims 1 to 7.
- A method of selectively controlling weeds in a field of plants according to either of claims 11 or 12, or seeds according to claim 13, comprising applying an effective
 amount of the herbicide and the exogenous inducer.

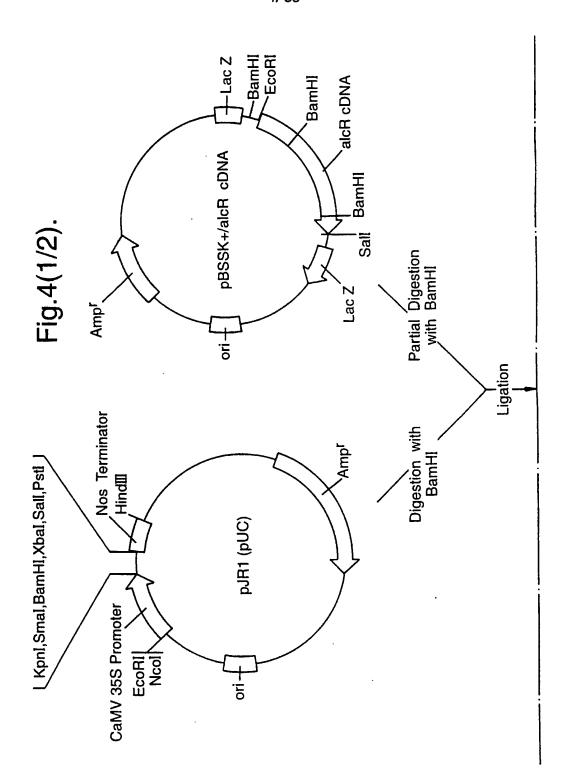


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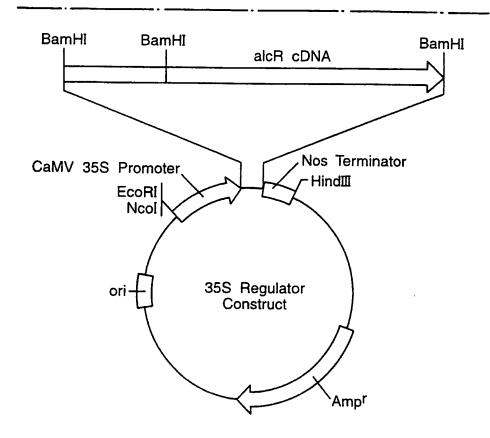


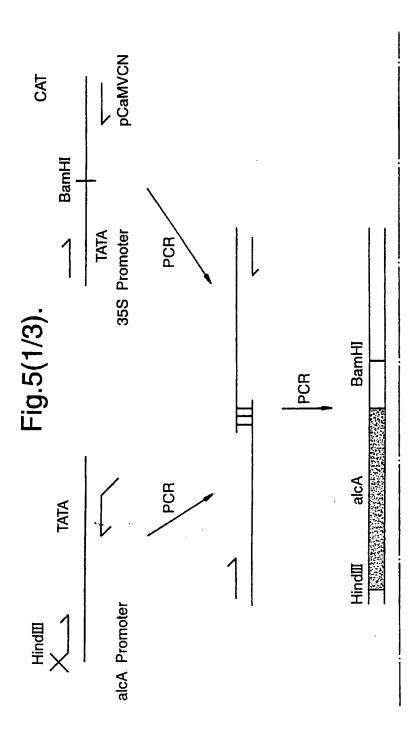
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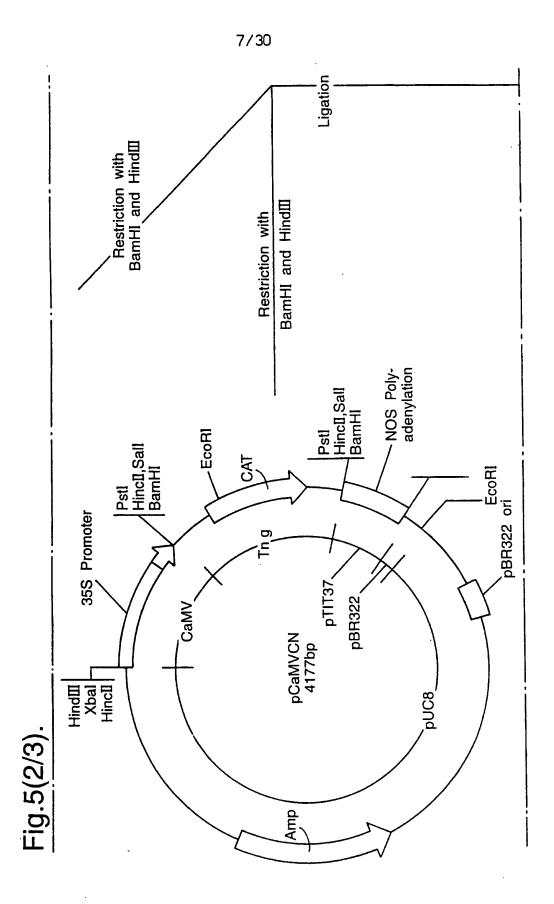


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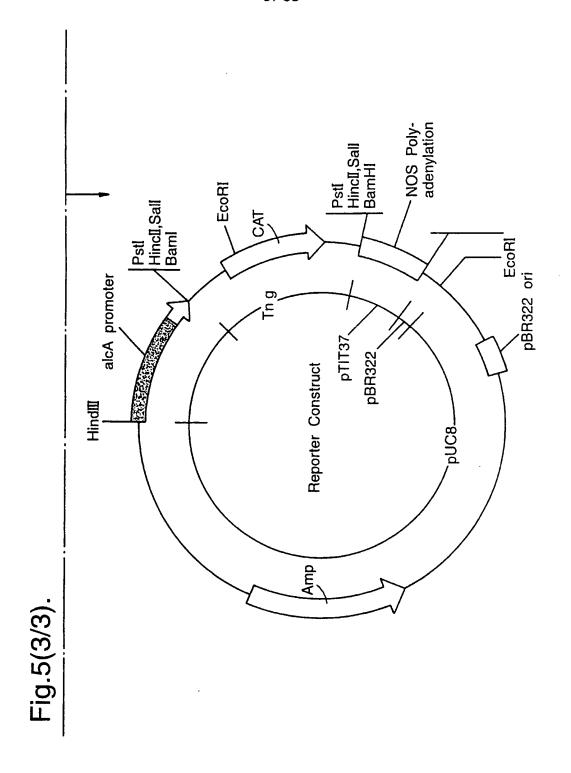
Fig.4(2/2).







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											OMOTER 4SE
	SON	SON	SON		SON	NOS	SON				QUITIN PRC
	сох	CP4	GOX		GOX	CP4	GOX	(3) A+B			UBI=MAIZE POLYUBIQUITIN PROMOTER AdHI=ALCOHOL DEHYDROGENASE RUBISCO) PETUNIA
DICOT	CTP1	CTP2	CTP1	MONOCOT	CTP1	CTP2	CTP1	(2) B	(5) B+C	(2) E+F	UBI=M AdHI=/ OPSIS RUBIS ASS 1. PETU
Q	TMW	TMW	TMW	MOL	IBN	UBI	AdHI				ICE 1 (ARABIC
	E35	E35	SWITCH		UB	UB	SWITCH	(-)	(4) C	(1) D+E	UBI=MAIZE NCER LAST TRANSIT SEQUENCE 1 (ARABIDOPSIS RUBISCO) LAST TRANSIT SEQUENCE EPSPS CLASS 1. PETUNIA JBIQUITIN PROMOTER
ii C	√	(a)	(a)		 _	Ш	<u>u</u>	TOBACCO		CORN	E 35=ENHANCED CAM TMV=TMV ENHANCER CTP1=CHLOROPLAST CTP2=CHLOROPLAST UB=MAIZE POLYUBIQU

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Fig.7(1/3).

10 20 30 40 50	
<u>1234567890 1234567890 1234567890 1234567890 1234567890</u>	
AAGCTTACCA TGGCTTCCTC TATGCTCTCT TCCGCTACTA TGGTTGCCTC K L T M A S S M L S S A T M V A S	50
TCCGGCTCAG GCCACTATGG TCGCTCCTTT CAACGGACTT AAGTCCTCCGPAQAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	100
CTGCCTTCCC AGCCACCCGC AAGGCTAACA ACGACATTAC TTCCATCACA A F P A T R K A N N D I T S I T	150
AGCAACGCCG GAAGAGTTAA CTGTATGCAG GTGTGGCCTC CGATTGGAAA S N G G R V N C M Q V W P P I G K	200
GAAGAAGITI GAGACICICI CITACCIICC IGACCITACC GATICCGGIG K K F E T L S Y L P D L T D S G G	250
GTCGCGTCAA CIGTATGCAG GCTATGGCTG AGAACCACAA GAAGGTTGGT R V N C M Q A M A E N H K K V G	300
ATCGCTGGAG CTGGAATCGT TGGTGTTTGC ACTGCTTTGA TGCTTCAACG I A G A G I V G V C T A L M L Q R	350
TCGTGGATTC AAGGTTACCT TGATTGATCC AAACCCACCA GGTGAAGGTG R G F K V T L I D P N P P G E G A	400
CTTCTTTCGG TAACGCTGGT TGCTTCAACG GTTCCTCCGT TGTTCCAATG S F G N A G C F N G S S V V P M	450
TCCATGCCAG GAAACTTGAC TAGCGTTCCA AAGTGGCTTC TGGATCCTGT S M P G N L T S V P K W L L D P V	500
TGTGAATTC V N	509

11/30 Fig.7(2/3).

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10 20 30 40 50	
1234567890 1234567890 1234567890 1234567890 1234567890	
AAGCTTACGG ATCCAATGGG TCCATTGTCC ATCCGTTTCA GCTACTTTCC	50
K L T D P M G P L S I R F S Y F P	
	100
AACCATCATG CCTTGGTTGA TTCGTTTCTT GCTTGCTGGA AGACCAAACA	100
TIM PWLI RFL LAG RPNK	
	150
AGGTGAAGGA GCAAGCTAAG GCACTCCGTA ACCTCATCAA GTCCACTGTG	100
V K E Q A K A L R N L I K S T V	
CCTTTGATCA AGTCCTTGGC TGAGGAGGCT GATGCTAGCC ACCTTATCCG	200
PLIKSLAEEADASHLIR	
TCACGAAGGI CACCTTACCG TGTACCGTGG AGAAGCAGAC TTCGCCAAGG	250
HEGHLTV YRGEAD FAKD	
	200
ACCGIGGAGG TIGGGAACTI CGICGICTCA ACGGIGTICG TACTCAAATC	300
RGG WEL RRLN GVR TQI	
	350
CTCAGCGCTG ATGCATTGCG TGATTTCGAT CCTAACTTGT CTCACGCCTT	
LSADALR DFD PNLS HAF	
TACCAAGGGA ATCCTTATCG AAGAGAACGG TCACACCATC AACCCACAAG	400
TKGILIE ENGHTINPQG	
GICTCGIGAC TCTCTTGTTT CGTCGTTTCA TCGCTAACGG TGGAGAGTTC	450
LVTLLF RRFI ANG GEF	
	500
GIGICIGCIC GIGITATCGG ATTCGAGACT GAAGGICGIG CICTCAAGGG	500
V S A R V I G F E T E G R A L K G	
	550
TATCACCACC ACCAACGGIG TICITGCIGI IGAIGCIGCA GIGITGIGAA	~~
I T T T N G V L A V D A A V L . I	
	553
TTC	

12/30 Fig.7(3/3).

10 20 30 40 50	
1234567890 1234567890 1234567890 1234567890	
AACCTTACTG CAGTTGTTGC AGCTGGTGCA CACTCCAAGT CTCTTGCTAA	50
K L T A V V A A G A H S K S L A N	
CTCCCTTGGT GATGACATCC CATTGGATAC CGAACGTGGA TACCACATCG	100
SLG DDIP LDT ERG YHIV	
TGATCGCCAA CCCAGAAGCT GCTCCACGTA TTCCAACTAC CGATGCTTCT	150
I A N P E A A P R I P T T D A S	100
IANFEAAINIIIDAO	
GGAAAGTTCA TCGCTACTCC TATGGAGATG GGTCTTCGTG TTGCTGGAAC	200
	200
G K F I A T P M E M G L R V A G T	
	250
CGTTGAGTTC GCTGGTCTCA CTGCTGCTCC TAACTGGAAG CGTGCTCACG	250
V E F A G L T A A P N W K R A H V	
	200
TTCTCTACAC TCGTGCTCGT AAGTTGCTTC CAGCTCTCGC TCCTGCCAGT	300
LYTRARKLLP ALA PAS	
	250
TCTGAAGAAC GTTACTCCAA GTGGATGGGT TTCCGTCCAA GCATCCCAGA	350
SEER YSK WMG FRPS I PD	
	400
TICCCTTCCA GIGATIGGIC GIGCTACCCG TACTCCAGAC GITATCTACG	400
SLP VIGR ATR TPD VIYA	
CITICGGICA CGGICACCIC GGIATGACIG GIGCICCAAT GACCGCAACC	450
FGHGHLGMTG APM TAT	
CICGITICIG AGCICCICGC AGGIGAGAAG ACCICIATCG ACATCICICC	500
LVSELLA GEK TSID I S P	
ATTCGCACCA AACCGTTTCG GTATTGGTAA GTCCAAGCAA ACTGGTCCTG	550
FAPNRFGIGKSKQ TGPA	
CATCCTAAGG TACCGAATTC	570
	3.0
S.GTEF	

				ATC EN		
	09	CTCCAAAAT GAGGTTTTTA	AAGGGTAATA TTCCCATTAT	GAAGATAGTG CTTCTATCAC	CATCGTTGAA GTAGCAACTT	CATCGTGGAA GTAGCACCTT
	50	AAGCTTGCAT GCTGCAGGT CAACATGGTG GAGCACGACA CACTTGTCTA CTCCAAAAAT TTCGAACGTA CGCACGTCCA GTTGTACCAC CTCGTGCTGT GTGAACAGAT GAGGTTTTTA	CAGTCTCAGA AGACCAAAGG GCAATTGAGA CTTTTCAACA AAGGGTAATA GTCAGAGTCT TCTGGTTTCC CGTTAACTCT GAAAAGTTGT TTCCCATTAT	TCCGGAAACC TCCTCGGATT CCATTGCCCA GCTATCTGTC ACTTTATTGT GAAGATAGTG AGGCCTTTGG AGGAGCCTAA GGTAACGGGT CGATAGACAG TGAAATAACA CTTCTATCAC	GAAAAGGAAG GTGGCTCCTA CAAATGCCAT CATTGCGATA AAGGAAAGGC CATCGTTGAA CTTTTCCTTC CACCGAGGAT GTTTACGGTA GTAACGCTAT TTCCTTTCCG GTAGCAACTT	GATGCCTCTG CCGACAGTGG TCCCAAAGAT GGACCCCCAC CCACGAGGAG CATCGTGGAA
Fig.8.	40	GAGCACGACA CTCGTGCTGT	GCAATTGAGA CGTTAACTCT	GCTATCTGTC CGATAGACAG	CATTGCGATA GTAACGCTAT	GGACCCCCAC
工	30	CAACATGGTG	AGACCAAAGG TCTGGTTTCC	CCATTGCCCA GGTAACGGGT	CAAATGCCAT GTTTACGGTA	TCCCAAAGAT AGGGTTTCTA
	O 20 Sphi Psti i	GCTGCAGGT	CAGTCTCAGA GTCAGAGTCT	TCCTCGGATT AGGAGCCTAA	GTGGCTCCTA CACCGAGGAT	CCGACAGTGG
	10 HindIII	1 AAGCTTGCAT GCCTGCAGGT TTCGAACGTA CGCACGTCCA	61 ATCAAAGATA CAGTCTCAGA AGACCAAAGG GCAATTGAGA CTTTTCAACA AAGGGTAATA TAGTTTCTAT GTCAGAGTCT TCTGGTTTCC CGTTAACTCT GAAAAGTTGT TTCCCATTAT	121 TCCGGAAACC TCCTCGGATT CCATTGCCCA GCTATCTGTC ACTTTATTGT GAAGATAGTG AGGCCTTTGG AGGAGCCTAA GGTAACGGGT CGATAGACAG TGAAATAACA CTTCTATCAC	181 GAAAAGGAAG GTGGCTCCTA CAAATGCCAT CATTGCGATA AAGGAAAGGC CATCGTTGAA CTTTTCCTTC CACCGAGGAT GTTTACGGTA GTAACGCTAT TTCCTTTCCG GTAGCAACTT	241 GATGCCTCTG CCGACAGTGG TCCCAAAGAT GGACCCCCAC CCACGAGGAG CATCGTGGAA CTACGAGAGAC GGTGTCACC AGGGTTTCTA CCTGGGGGTG GGTGCTCTC GTAGCACCTT
		-	61	121	181	241

Fig.8 (Cont).

		<i></i>	35S PR			
ACATGGTGGA Y	A CCAAAGGGC	ATTGCCCAGC	AATGCCATCA	CCAAAGATGG	TCTTCAAAGC	CACTATCCTT
	TGGTTTCCCG	TAACGGGTCG	TTACGGTAGT	GGTTTCTACC	AGAAGTTTCG	GTGATAGGAA
AAAAGAAGAC GTTCCAACCA CGTCTTCAAA GCAAGTGGAT TGATGTGATA ACATGGTGGA	GCACGACACA CTTGTCTACT CCAAAATAT CAAAGATACA GTCTCAGAAG ACCAAAGGGC	421 AATTGAGACT TTTCAACAAA GGGTAATATC CGGAAACCTC CTCGGATTCC ATTGCCCAGC	TATCTGTCAC TTTATTGTGA AGATAGTGGA AAAGGAAGGT GGCTCCTACA AATGCCATCA	TIGCGATAAA GGAAAGGCCA TCGTTGAAGA TGCCTCTGCC GACAGTGGTC CCAAAGATGG	601 ACCCCCACCC ACGAGGAGCA TCGTGGAAAA AAGAAGACGT TCCAACCACG TCTTCAAAGC	AAGTGGATTG ATGTGATATC TCCACTGACG TAAGGGATGA CGCACAATCC CACTATCCTT TTCACCTAAC TACACTATAG AGGTGACTGC ATTCCCTACT GCGTGTTAGG GTGATAGGAA
TTTTCTTCTG CAAGGTTGGT GCAGAAGTTT CGTTCACCTA ACTACACTAT TGTACCACCT	CGTGCTGTGT GAACAGATGA GGTTTTTATA GTTTCTATGT CAGAGTCTTC TGGTTTCCCG	TTAACTCTGA AAAGTTGTTT CCCATTATAG GCCTTTGGAG GAGCCTAAGG TAACGGGTCG	ATAGACAGTG AAATAACACT TCTATCACCT TTTCCTTCCA CCGAGGATGT TTACGGTAGT	AACGCTATTT CCTTTCCGGT AGCAACTTCT ACGGAGACGG CTGTCACCAG GGTTTCTACC	TGGGGGTGGG TGCTCCTCGT AGCACCTTTT TTCTTCTGCA AGGTTGGTGC AGAAGTTTCG	
GCAAGTGGAT	CAAAGATACA	CGGAAACCTC	AAAGGAAGGT	TGCCTCTGCC	AAGAAGACGT	TAAGGGATGA
CGTTCACCTA	GTTTCTATGT	GCCTTTGGAG	TTTCCTTCCA		TTCTTCTGCA	ATTCCCTACT
CGTCTTCAAA	CCAAAAATAT	GGGTAATATC	AGATAGTGGA	GGAAAGGCCA TCGTTGAAGA TGCCTCTGCC	TCGTGGAAAA	ATC TCCACTGACG TAG AGGTGACTCC
GCAGAAGTTT	GGTTTTTATA	CCCATTATAG	TCTATCACCT	CCTTTCCGGT AGCAACTTCT ACGGAGACGG	AGCACCTTTT	
GTTCCAACCA	CTTGTCTACT	TTTCAACAA	TTTATTGTGA	GGAAAGGCCA	ACGAGGAGCA	ATGTGATATC
CAAGGTTGGT	GAACAGATGA	AAAGTTGTTT	AAATAACACT	CCTTTCCGGT	TGCTCCTCGT	TACACTATAG
	GCACGACACA	AATTGAGACT TTAACTCTGA		TTGCGATAAA AACGCTATTT	ACCCCCACCC TGGGGGTGGG	
301	361	421	481	541	601	661

Fig. 8 (Cont i)

TSP Xhol

NOS 3, ₽a TGAGATGGGT ACTCTACCCA TTTACAATTA AAATGTTAAT TTTGGCAATA AAATATAGCG TTTATATCGC AATTTCTGTT GGAGAGG TCGAGTATTT ccrcrccrde accrdarana AAACCGTTAT TTAAAGACAA GGGAATTC ACAATTACTA TGTTAATGAT GTCATCTATG TTACTAGATC CAGTAGATAC AATGATCTAG GCAAGTTTGT TAATAGTATA ACGTTATTTA TGCAATAAAT TATCTTTGT CGTTCAAACA ATTATCATAT ATAGAAACA TCTTGCGATG AGAACGCTAC GTAATGCATG CATTACGTAC TACCAACAAC AACAAACAAC AAACAACATT ATGGTTGTTG TTGTTGTTG TTGTTGTAA Smal Konl Sacl CACCATGGAT CCCCGGTAC GGAGCTGGAA TTTCCCCGAT GTGGTACCTT AAAGGGGCTA TTAATACGCG TTAATATGTA AATTATGCGC AAGGAGATAT ATTCCTTCAA GTAAAGTAAA TICCICIATA TANGGAGGIT CATITICATIT CTGTTGCCGG TAATTAACAT ATTAATTGTA AATTATACAT CTATTTAATA GCGCGCGCCA CGCGCGCGT TATA BOX AGAGTCCCGC 1 AAGTTTCTTA AGATTGAATC TTCAAAGAAT TCTAACTTAG AAGCATGTAA TTCGTACATT GATAAATTAT GAATTACGTT CTTAATGCAA TTTATGATT AAAATACTAA CGCAAACTAG AATGTTGTTA GCGTTCTGGG CGCAAGACCC TTACAACAAT 1021 781 901 961 1081 721 841

Total number of bases is: 1138. DNA sequence composition: 370 A; 253 C; 234 G; 281 T;

OTHER;

0

EcoRi

Sequence name: PMJB1

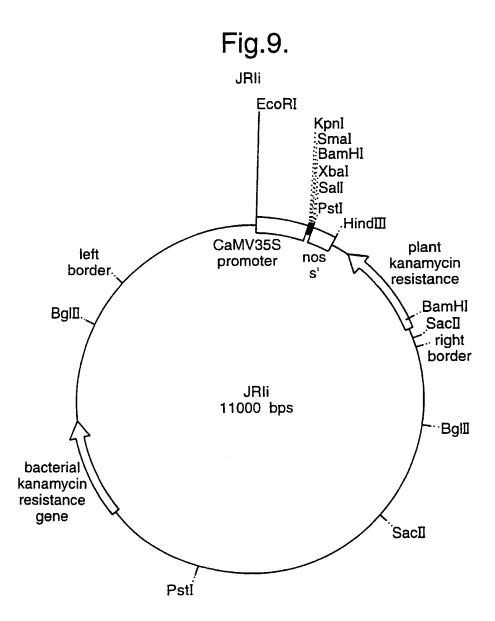


Fig.10(1/4).

10	20	30	40	50	
1234567890	1234567890	1234567890 1	.234567890	1234567890	
		TAGCAGAATC T			50
K L T M	A Q V	S R I C	N G V	Q N P	
		CGAAATCCAG T K S S			100
		CAGCATCCAC G Q H P R			150
		TGGGATGACG T G M T L			200
TCCTCTTAAG P. L K	GICATGICTT V M S S	CTGTTTCCAC G	ECCTCTATC A C M	CTTCACGGTG L H G A	250
CAAGCAGCCG S S R	TCCAGCAACT P A T	GCTCGTAAGT C	CICIGGICI S G L	TTCTGGAACC S G T	300
GICCGTATIC V R I P	CAGGTGACAA G D K	GTCTATCTCC C	CACAGGICCT H R S F	TCATGITIGG M F G	350
AGGTCTCGCT G L A	AGCGGTGAAA S G E T	CTCGTATCAC C	CESTCITITIS G L L	GAAGGTGAAG E G E D	400
		GCTATGCAAG C		CAGGATCCIG R I L	450
TIGIGAATIC L . I					460

Fig. 10(2/4). 18/30

10 20 30 40 50	
1234567890 1234567890 1234567890 1234567890 1234567890	
AAGCTTAGGA TCCGTAAGGA AGGTGATACT TGGATCATTG ATGGTGTTGG	50
KLRIRKE GDT WIID GVG	
TAACGGTGGA CTCCTTGCTC CTGAGGCTCC TCTCGATTTC GGTAACGCTG	100
NGGLLAPEAPLDFGNAA	
CAACTGGTTG CCGTTTGACT ATGGGTCTTG TTGGTGTTTA CGATTTCGAT	150
TGC RLT MGLV GVY DFD	
AGCACTITCA TIGGIGACGC TICTCICCACT AAGCGICCAA IGGGICGIGI	200
S T F I G D A S L T K R P M G R V	
GTTGAACCCA CTTCGCGAAA TGGGTGTGCA GGTGAAGTCT GAAGACGGTG	250
LNPLREMGVQVKSEDGD	
ATCGTCTTCC AGTTACCTTG CGTGGACCAA AGACTCCAAC GCCAATCACC	300
R L P V T L R G P K T P T P I T	200
TACAGGTAC CTATGCTTC CGCTCAAGTG AAGTCCGCTG TTCTGCTTGC	350
Y R V P M A S A Q V K S A V L L A	
TGGTCTCAAC ACCCCAGGTA TCACCACTGT TATCGAGCCA ATCATGACTC	400
G L N T P G I T T V I E P I M T R	
GTGACCACAC TGAAAAGATG CTTCAAGGTT TTGGTGCTAA CCTTACCGTT	450
D H T E K M L O G F G A N L T V	450
GAGACTGATG CTGACGGTGT GCGTACCATC CGTCTTGAAG GTCGTGGTAA	500
ETDADGVRTIRLEGRGK	
GCTCACCGGT CAAGTGATTG ATGTTCCAGG TGATCCATCC TCTACTGCTT	550
L T G Q V I D V P G D P S S T A F	
	600
TCCCATTGGT TGCTGCCTTG CTTGTTCCAG GTTCCGACGT CACCATCCTT P L V A A L L V P G S D V T I L	600
AACGTTTTGA TGAACCCAAC COGTACTGGT CTCATCTTGA CTCTGCAGTG	650
N V L M N P T R T G L I L T L Q C	

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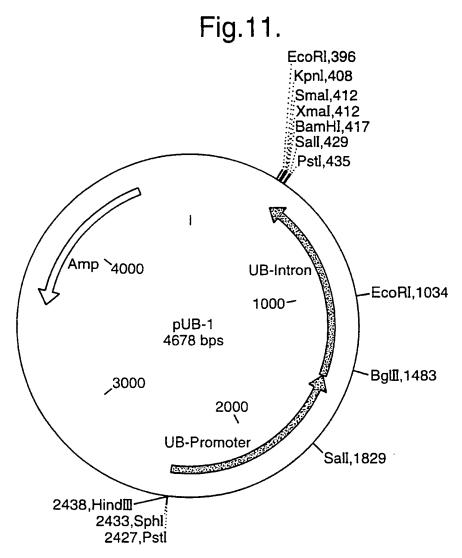
Fig.10(3/4).

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
TIGIGAATIC					660
C = E					

20/30

Fig.10(4/4).

10 20 30 40 50	
1234567890 1234567890 1234567890 1234567890 1234567890	
AAGCTTCTGC AGGAAATGGG TGCCGACATC GAAGTGATCA ACCCACGTCT	50
K L L Q E M G A D I E V I N P R L	
TGCTGGTGGA GAAGACGTGG CTGACTTGCG TGTTCGTTCT TCTACTTTGA	100
AGGEDVA DLR VRS STLK	
AGGGTGTTAC TGTTCCAGAA GACCGTGCTC CTTCTATGAT CGACGAGTAT	150
G V T V P E D R A P S M I D E Y	100
CCAATTCTCG CTGTTGCAGC TGCATTCGCT GAAGGTGCTA CCGTTATGAA	200
PILAVAAAFAEGATVMN	200
CGGTTTGGAA GAACTCCGTG TTAAGGAAAG CGACCGTCTT TCTGCTGTCG	250
G L E E L R V K E S D R L S A V A	250
G L E E L K V K E S D K L S A V A	
CAAACGGTCT CAAGCTCAAC GGTGTTGATT GCGATGAAGG TGAGACTTCT	300
	300
NGL KLN G V D C D E G E T S	
CTCGTCGTGC GTGGTCGTCC TGACGGTAAG GGTCTCGGTA ACGCTTCTGG	350
	350
L V V R G R P D G K G L G N A S G	
ACCACCTORS comacocatos mocatros manocomanos acommocinos	400
AGCAGCTGTC GCTACCCACC TCGATCACCG TATCGCTATG AGCTTCCTCG	400
AAV ATHL DHR IAM SFLV	
	450
TTATGGGTCT CGTTTCTGAA AACCCTGTTA CTGTTGATGA TGCTACTATG	450
M G L V S E N P V T V D D A T M	
1	500
ATCGCTACTA GCTTCCCAGA GTTCATGGAT TTGATGGCTG GTCTTGGAGC	500
I A T S F P E F M D L M A G L G A	
TAAGATCGAA CTCTCCGACA CTAAGGCTGC TTGATGAGCT CGAATTC	547
KIE LSDT KAA A RI	



Ubiquitin promoter fragment PCRed from maize. 2 Kb. fragment cloned into pUC19. Junctions have been sequenced to confirm that it is the Ubiquitin promoter.

Fig.12.

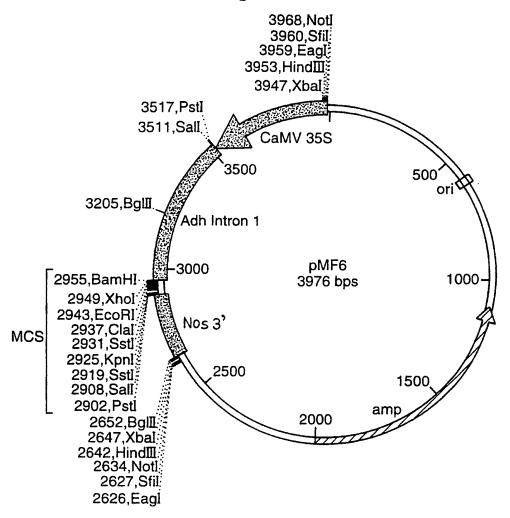


Fig.13.

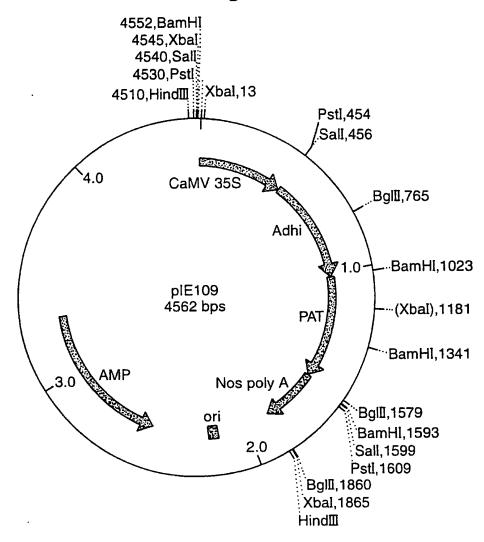


Fig.14.

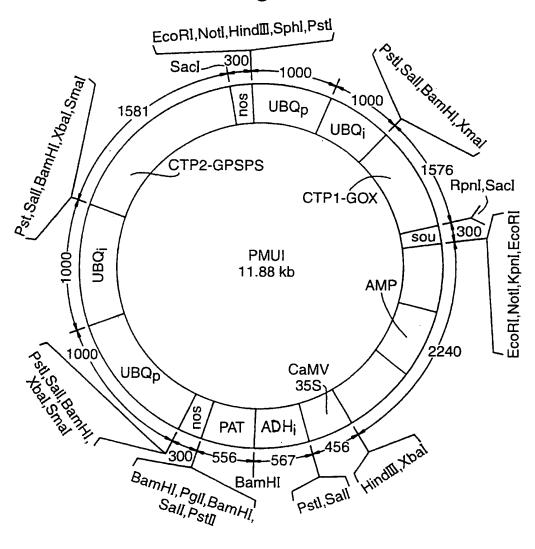
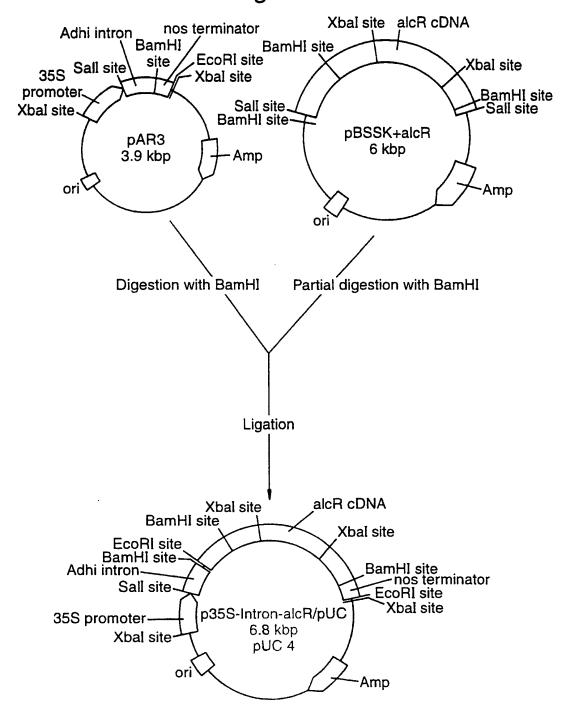
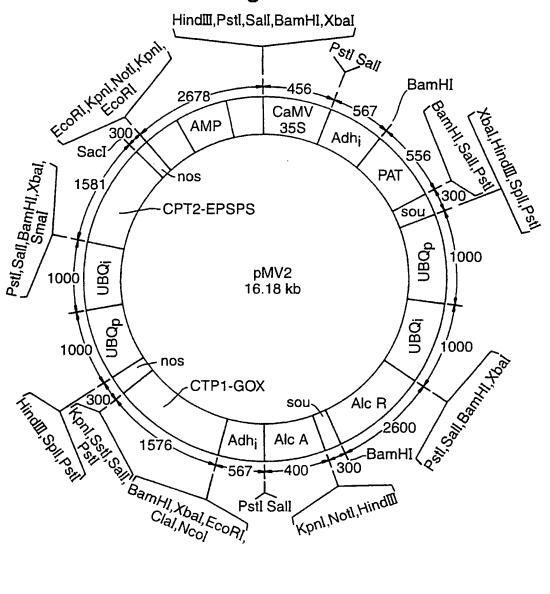


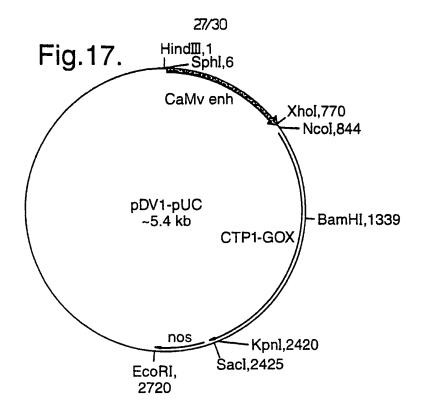
Fig.15.

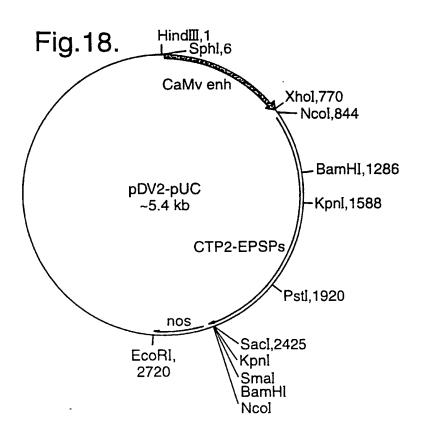


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Fig.16.

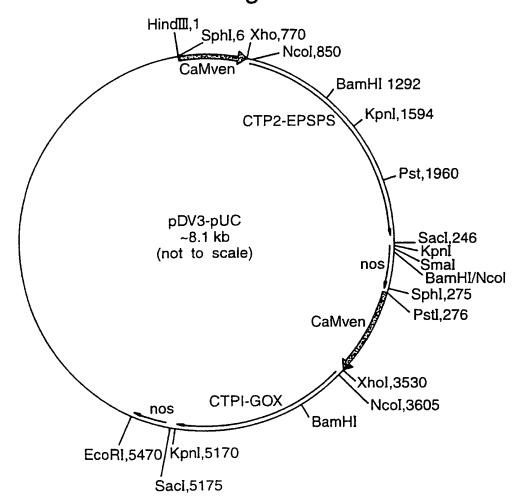


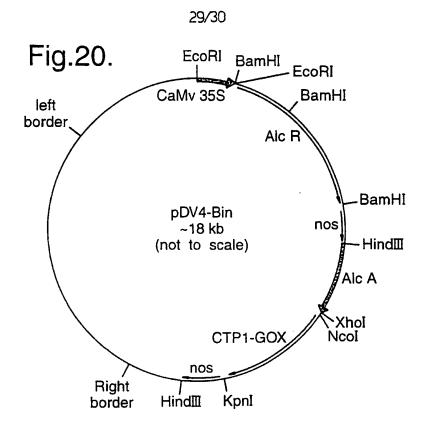


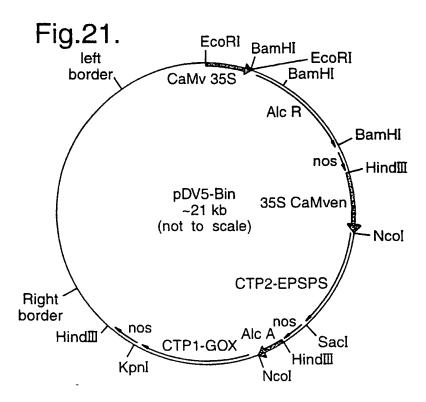


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Fig.19.

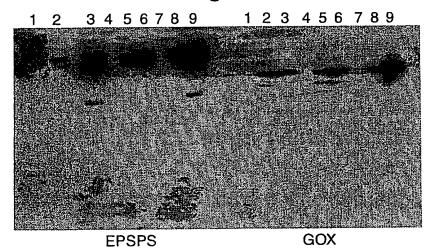






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Fig.22.



INTERNATIONAL SEARCH REPORT

national Application No PCT/GB 96/01883

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/82 C12N5/10 A01H5/00 According to International Patent Classification (IPC) or to both national classification and IPC **B. FIELDS SEARCHED** Minimum documentation searched (classification system followed by classification symbols) C12N A01H IPC 6 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practical, search terms used) C. DOCUMENTS CONSIDERED TO BE RELEVANT Relevant to claim No. Citation of document, with indication, where appropriate, of the relevant passages 1,3, EP,A,O 388 186 (DU PONT) 19 September 1990 Χ 7-12,14, 15 3 see page 10, line 35 - line 41 Y see page 78, line 1 - page 82, line 40 3 WO,A,93 01294 (ICI PLC) 21 January 1993 Υ see page 11, line 16 - page 12, line 2 1.3.8-12 WO,A,93 05164 (UNIV LEICESTER) 18 March X 1993 see page 54, line 10 - line 18 see page 48, line 1 - line 19 -/--Patent family members are listed in annex. Further documents are listed in the continuation of box C. later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to filing date involve an inventive step when the document is taken alone "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "O" document referring to an oral disclosure, use, exhibition or *P* document published prior to the international filing date but later than the priority date claimed '&' document member of the same patent family Date of mailing of the international search report Date of the actual completion of the international search 2 9. 01. 97 15 January 1997 Authorized officer Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentiaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Maddox, A Fax: (+31-70) 340-3016

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fr national Application No

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